New approaches for flavoenzyme applications

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CHAPTER 1

INTRODUCTION

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1 FLAVOENZYMES

1.1 FLAVIN COFACTORS

Flavoenzymes are enzymes that depend on a flavin cofactor for their function. They are expressed in every kind of living organisms. The most common flavin cofactor is flavin adenine dinucleotide (FAD), which typically binds to the target flavoenzyme with very high affinity. FAD contains a ribityl chain to which two phosphate moieties and an adenosine are attached at one side, and at the other side it is linked to the isoalloxazine ring (Figure 1). The typical, tricyclic isoalloxazine ring is responsible for the yellow-orange color of this molecule and determines its redox properties (Kamerbeek, 2004)

In living cells the biosynthesis of FAD-containing flavoenzymes is a three-step process, in which vitamin B2 (riboflavin, Figure 1) is a precursor. Riboflavin is phosphorylated by the riboflavin kinase into flavin mononucleotide (FMN). Subsequently, FMN is adenylated by FAD synthetase and forms FAD. Once the FAD cofactor is present in the cell, its incorporation into the flavoprotein active center is typically spontaneous due to the high affinity towards this ligand.

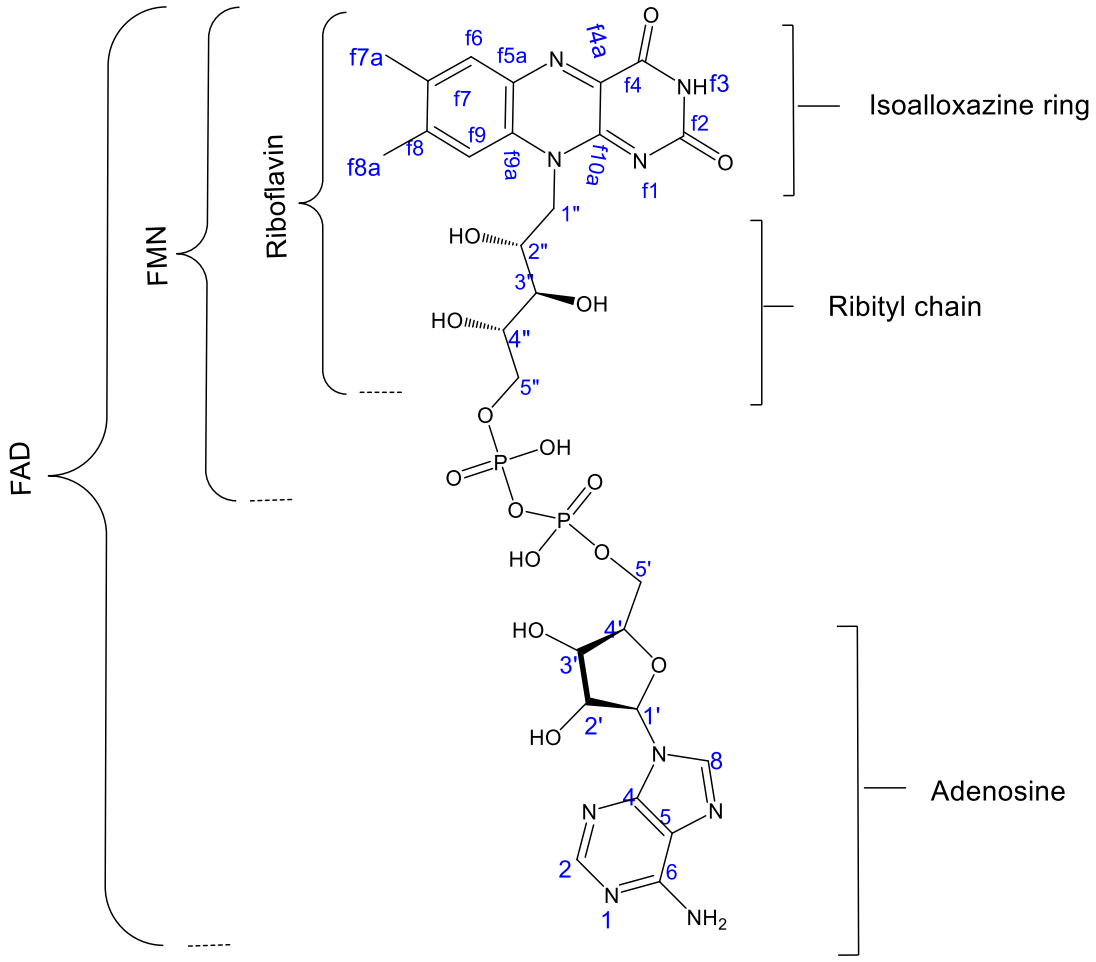

***Figure 1**. Structural formula of FAD with indicated subcomponents and atom numbering.*

The minority (around 10%) of flavoenzymes bind FAD covalently (Macheroux, Kappes and Ealick, 2011). The covalent binding most commonly involves the f8a-methyl or f6 carbon atoms of the isoalloxazine ring (Jin *et al.*, 2008; Heuts *et al.*, 2009) (Figure 1) and alters the redox properties of the molecule (Heuts *et al.*, 2009). For example, it was reported that after removal of the histidyl-FAD bond in vanillyl alcohol oxidase (VAO) from *Penicillium simplicissimum*, the redox potential drops by 0.12 V (Jin *et al.*, 2008).

1.2 Classification of FAD-containing enzymes

Regarding catalytic activity, FAD-containing flavoenzymes can be classified based on their mechanism of action, type of chemical reaction that is catalyzed or the type of reducing/oxidizing substrates used during conversion (Massey, 2000). The most common classification is based on the types of chemical reactions catalyzed. By such discrimination, there are a few classes defined: oxidases, dehydrogenases/reductases, disulfide oxidoreductases, monooxygenases and non-redox flavoenzymes. Such a diverse activity of enzymes is observed due to different microenvironments of their active centers, that are tuned by the amino acid side chains that encompass the isoalloxazine ring of the FAD cofactor. Below, two groups of oxidative flavoenzymes are introduced as they are most relevant to the rest of the thesis: the FAD-containing flavoprotein monooxygenases and oxidases. Both enzyme groups are also intensely studied because of their potential for biotechnological applications.

1.2.1 Flavoprotein monooxygenases

Incorporation of one oxygen atom into organic molecules defines the activity of flavoprotein monooxygenases (Chaiyen, Fraaije and Mattevi, 2012). Catalysis starts with oxygen activation by a reaction of dioxygen with the f4a moiety of a reduced flavin cofactor which results in formation of a flavin peroxide (Figure 2). The peroxyflavin is an intermediate, which in Baeyer-Villiger Monooxygenases (BVMOs) can be stable up to minutes (Torres Pazmiño et al., 2008), and in the so-called flavin-containing monooxygenases (FMOs) can last for hours (Krueger and Williams, 2005). This intermediate performs the direct oxygenation of a substrate molecule in the active center. Upon insertion of one oxygen into substrate, the second oxygen atom is released by formation of water. To perform the next oxygenation, a monooxygenase needs to recharge its flavin cofactor with electrons. The natural electron donors are reduced nicotinamide cofactors: flavoprotein monooxygenases are NAD(P)H-dependent (Torres Pazmiño and Fraaije, 2008). An example of a reaction catalyzed by a FAD-containing and NADPH dependent flavoprotein monooxygenase is shown in Figure 3: the Baeyer-Villiger oxidation of phenylacetone by phenylacetone monooxygenase (PAMO). This flavoenzyme was used as prototype flavoprotein monooxygenase in Chapters 2 and 3 of this thesis.

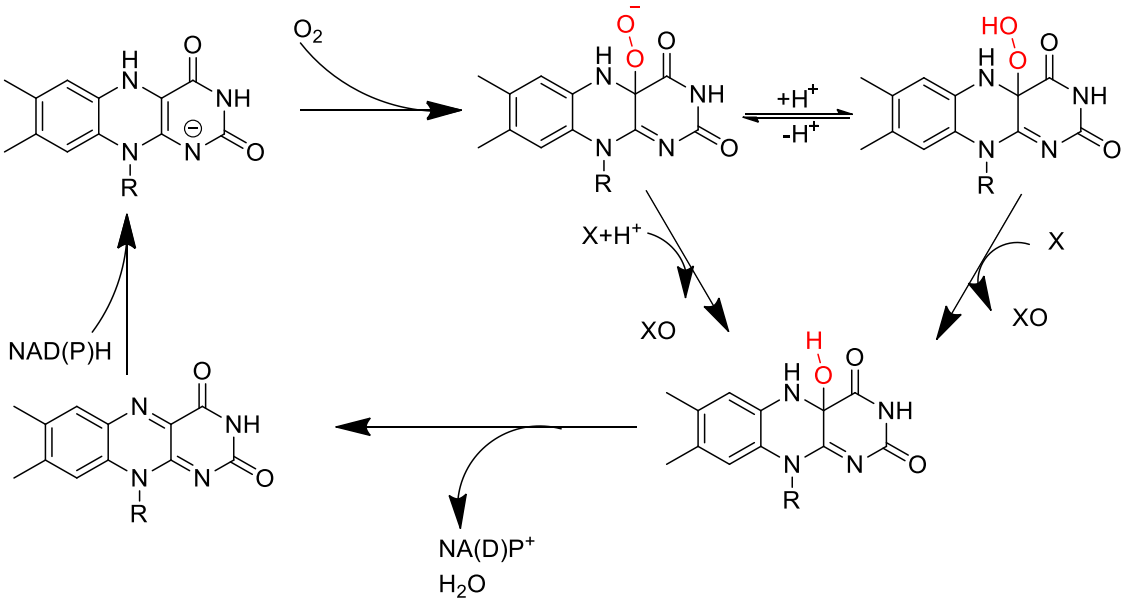

Figure 2. *Catalytic cycle of flavoprotein monooxygenases. The scheme is based on (Torres Pazmiño et al., 2008) and may vary depending on the respective flavoprotein monooxygenase.*

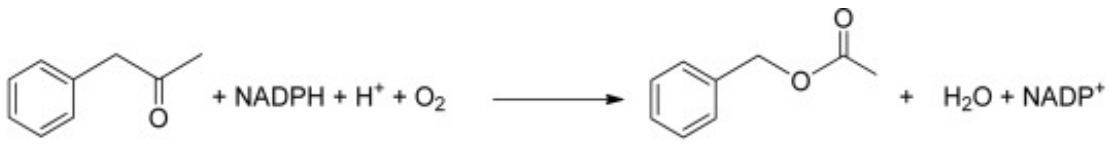

Figure 3*. Schematic representation of the reaction catalyzed by phenylacetone monooxygenase (PAMO): a Baeyer-Villiger oxidation of phenylacetone into benzyl acetate.*

The human proteome contains several flavoprotein monooxygenases. The most intensively studied are the so-called human flavin-containing monooxygenases (hFMOs) due to their considerable contribution to the detoxification of xenobiotics (Motika, Zhang and Cashman, 2007). They catalyze 10% of the oxidative metabolism in liver by oxidation of soft nucleophilic heteroatoms like sulfur or nitrogen (Cashman and Zhang, 2006). They act on drugs like lidocaine, amphetamine and albendazole. Human FMOs are FAD-containing, microsomal flavoenzymes and show some sequence homology with BVMOs such as PAMO. The human proteome contains five FMO isoforms (hFMO1-5), each isoform is encoded by a different gene. Human FMO isoform 3 (hFMO3) is predominantly expressed in human liver and is considered to be the most important hFMO concerning human xenobiotic metabolism (Cashman and Zhang, 2006). Till now, efficient recombinant overexpression of native hFMO3 has not been achieved and isolation of the enzyme or close homologs from mammalian tissue is troublesome, which has hampered detailed studies of this important human enzyme. Chapter 4 provides and overview on the current status on recombinant hFMO3 expression and our attempts to produce the enzyme as fusion enzyme using a bacterial expression host. Chapter 5 offers an alternative

approach for generating hFMO-related metabolites. We demonstrate that microbial sequence-related flavoprotein monooxygenases can be used as mimics of hFMO3 and its isoforms.

1.2.2 FLAVOPROTEIN OXIDASES

Flavoprotein oxidases catalyze the oxidation of various organic compounds. Based on sequence- and structural homology, six different flavoprotein oxidases can be identified (Dijkman *et al.*, 2013). The most common mechanism for oxidizing their substrate involves a direct hydride transfer (Kamerbeek, 2004). After oxidation, the reduced flavin cofactor donates its electrons to molecular oxygen yielding hydrogen peroxide. The largest flavoprotein oxidase family, the GMC family, includes some of the most studied and widely applied oxidases, such as the FAD-containing glucose oxidase and methanol oxidase. Another fairly widely distributed family of flavoprotein oxidases is named after VAO from the fungus *Penicillium simplicissimum*. VAO is able to catalyze the oxidation of various phenolic compounds. The oxidation of vanillyl alcohol leads to the known flavor compound vanillin, which is the main component of the natural vanilla flavor (Figure 4). The VAO family of FAD-containing oxidases is relatively rich in flavoprotein oxidases that carry a covalently attached FAD cofactor. For evaluating the use of the N^6-modified FAD derivative as anchor for enzyme immobilization as described in Chapters 2 and 3, a recently discovered VAO homolog (eugenol oxidase, EUGO) was used.

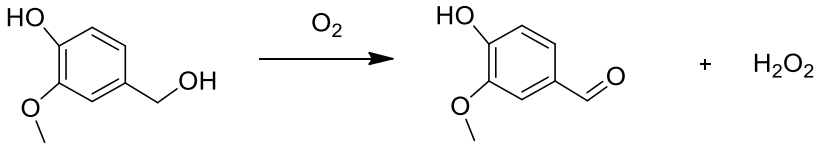

Figure 4. *A typical reaction catalyzed by VAO and EUGO: the oxidation of vanillin alcohol.*

2. APO FLAVOENZYME PRODUCTION

Flavoenzymes typically contain a tightly bound flavin cofactor. For many purposes it can be very useful to have access to a flavoenzyme devoid of its flavin cofactor, in its so-called apo form. Apo flavoenzymes are often prepared with the aim to determine their cofactor affinity. In other experiments apo flavoenzyme have been used for their reconstitution with an artificial cofactor in order to explore new enzymatic properties and applications. Deflavinylation techniques aim to produce the apo form of a flavoprotein which is still in the folded state, competent of binding the cofactor again. Reconstitution of properly prepared apo enzyme should yield a fully active holoenzyme. Most of the described approaches are only applicable to non-covalent FAD- or FMN-dependent flavoenzymes (Hefti,

Vervoort and Van Berkel, 2003). Nevertheless, with some newly developed approaches it is also possible to prepare the apo form for covalent flavoenzymes.

2.1 NON-COVALENT FLAVOENZYMES

For non-covalent flavoenzymes, deflavinylation is typically achieved upon (partial) unfolding enzyme, as it results in release of the flavin cofactor. This process requires mild conditions in order to prevent irreversible deactivation/unfolding (Hefti, Vervoort and Van Berkel, 2003). Usually, the addition of urea, guanidinium hydrochloride, halide ions and/or the use of ammonium sulfate results in deflavinylation. Optimization of the deflavinylation solution usually involves careful balancing the concentration of unfolding agent(s), salt(s) and pH. Glycerol might be added to protect the protein from full denaturation. Reversible immobilization of the target holo protein during the deflavinylation step can assist in this procedure. This has as benefit that the immobilized enzyme tends to be more stable, which is crucial during the application of the deflavinylation buffer, and the immobilized enzyme will not be able to form aggregate with other protein molecules. Furthermore, removal of the flavin cofactor from the immobilized protein can be easily monitored because it can simply be seen whether the yellow cofactor leaves the column. A deflavinylation procedure which involves the use of nickel-Sepharose to bind His-tagged flavoproteins is shown in Figure 5. Despite many trials, some proteins seem to be resilient towards any deflavinylation protocol indicating that preparation of apo flavoproteins can be a challenge (Hefti, Vervoort and Van Berkel, 2003)

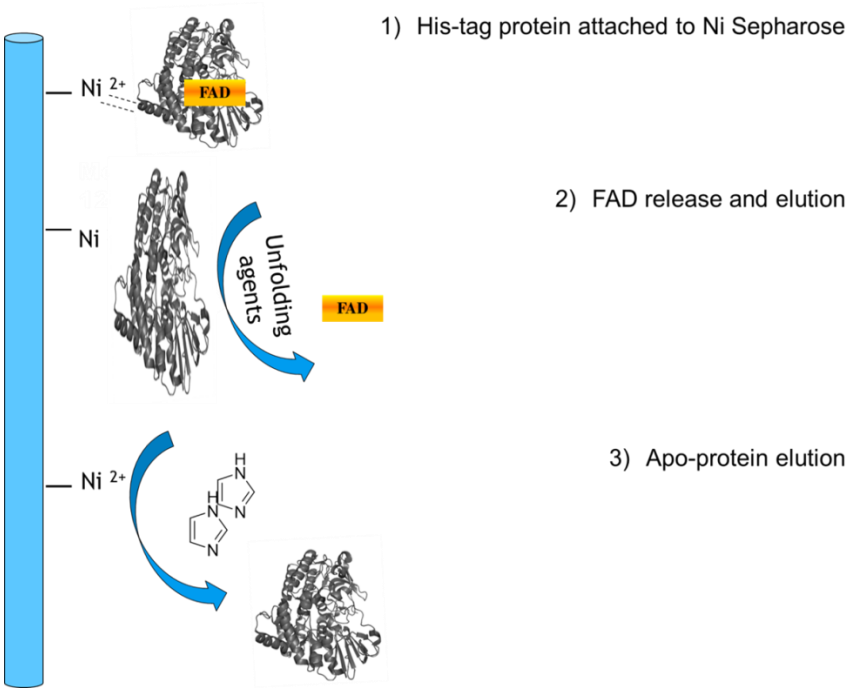

***Figure 5**. Deflavinylation using a nickel-Sepharose column (Hefti, Vervoort and Van Berkel, 2003)*

2.2 COVALENT FLAVOENZYMES

A generic method for deflavinylation has also been developed for the preparation of the apo form of a covalent flavoproteins. For that purpose, riboflavin-auxotrophic yeast and bacterial strains have been employed. By using such strains that are unable to synthesize the mature form of the common flavin cofactors, FMN and FAD, production of apo protein is feasible. For this approach, an effective expression vector is required that can be used in the available riboflavin-auxotrophic strains. The method is rather straightforward and has been successfully demonstrated for several covalent flavoproteins (Hefti, Vervoort and Van Berkel, 2003; Vogl *et al.*, 2007). However, it has as a disadvantage that the amount of produced apo protein is often rather low and often still contains a significant fraction of holo protein.

3. APPROACHES FOR ENZYME IMMOBILIZATION

Protein immobilization techniques have been developed since the 17th century (Cao, 2006). A huge advantage of immobilized proteins is that they can be easily separated from the reaction mixture and reused. Besides that, immobilization yields a high local concentration of enzymes, which is space-efficient. Nevertheless, when the enzymes are densely packed, the protein microenvironment can change, and it may alter enzymes' specificity, stability and kinetic parameters (Brena, González-Pombo and Batista-Viera, 2006). In fact, immobilization usually results in more stable proteins toward elevated temperatures and/or organic solvents. Additionally, immobilized enzymes can be used to perform reactions in atypical media and in a continuous mode, which allows their application in flow systems.

Enzymes can be immobilized by physical non-covalent interactions or in a covalent manner. Besides those two main approaches, there are also cross-linking techniques which result in enzymatic molecules attached one to another or being encapsulated (Brena, González-Pombo and Batista-Viera, 2006).

Covalent immobilization techniques usually employ an activated carrier, which subsequently can be linked to the target enzyme via functional groups localized at the protein surface. The carrier is typically functionalized with amine, carbonyl, hydroxyl or thiol groups or (very rarely) with the prosthetic group of a target enzyme. To create the covalent attachment of an enzyme usually relatively harsh conditions are applied. This often partially or fully inactivates the enzyme of interest. Therefore, optimization of covalent immobilization usually focuses on trying to create conditions that preserve enzyme activity during this process. Covalent immobilization, depending on the orientation of an enzyme molecule towards the carrier, can be divided into two groups: random and oriented.

3.1 RANDOM COVALENT ENZYME IMMOBILIZATION

Random immobilization methods typically exploit the reactivity of amino acids that are on the surface of a protein. Therefore it is applicable for virtually any enzyme. Because the generic nature of the

immobilization method, pure enzyme is required in case contamination with another biocatalysts has to be prevented. While random covalent immobilization is often employed because of its rather facile methodology, it has several disadvantages. The activity of randomly immobilized enzymes often can suffer from steric hindrances, causing limited mass transfer rates and sometimes (partial) loss of enzyme activity due to collapse of the enzyme molecular structure. Moreover, the covalent approach typically results in multiple enzyme layers on the carrier material (Cao, 2006). The latter phenomenon can be beneficial as results in a high enzyme loading. However, at the same time, it is may result in efficient usage of the immobilized enzyme due to limited mass transfer or enzyme deactivation.

Table 1. *Characteristics of standard covalent immobilization techniques (Brena, González-Pombo and Batista-Viera, 2006)*

Covalent immobilization	
Advantages	**Disadvantages**
High rigidity	Decrease in activity
Increased enzyme stability	High purity of enzyme is required during immobilization
High reusability	Change of enzyme characteristics (kinetics, specificity)
Suitable to scale up	Limited mass transfer

3.2. ORIENTED (COVALENT) ENZYME IMMOBILIZATION

Oriented immobilization, in which the site and type of attachment is targeted, usually results in formation of enzyme monolayers on the carrier surface with a tunable spatial distribution (Cao, 2006). This approach has higher chances to preserve full enzymatic activity. It typically depends on the design of a reactive ligand that is recognized by the target enzyme and can form a tight or even covalent complex.

A specific example of the oriented immobilization approach is to employ a cofactor for the attachment of a particular cofactor-binding enzyme to the carrier. This approach has been demonstrated already in 70's. At that time it served also to design affinity chromatography, specific for particular group of enzymes. One example is the immobilization of NAD^+ on reactive Sepharose material for the selective immobilization of the NAD-dependent dehydrogenases (Barry and O'Carra, 1973). Another interesting example was the preparation of Sepharose material functionalized with the cofactor pyridoxal 5'-phosphate. This could be used for successful immobilization of tyrosinase and tryptophanase (Goldstein and Katchalski-Katzir, 1976). The same immobilization approach can be employed to the FAD cofactor (Figure 6). As FAD can selectively bind to apo flavoenzymes with very

high affinity, this procedure does not require a high purity of the protein sample. Immobilization via the FAD cofactor has been demonstrated for a few times in the past few decades (Wingard, 1984). Nevertheless, this approach depends on a flavoenzyme which is able to bind a FAD cofactor to which a linker has been attached. In some cases, protein engineering may be needed to introduce such cofactor binding property. Nowadays, a large number of flavoenzymes have been described in detail, including their molecular structures, and plenty techniques for their engineering are available (van Berkel, Kamerbeek and Fraaije, 2006). Chapter 2 describes the successful FAD-mediated immobilization of PAMO and PAMO fused to phosphite dehydrogenase. We could show that the developed method results in a stable and fully active PAMO.

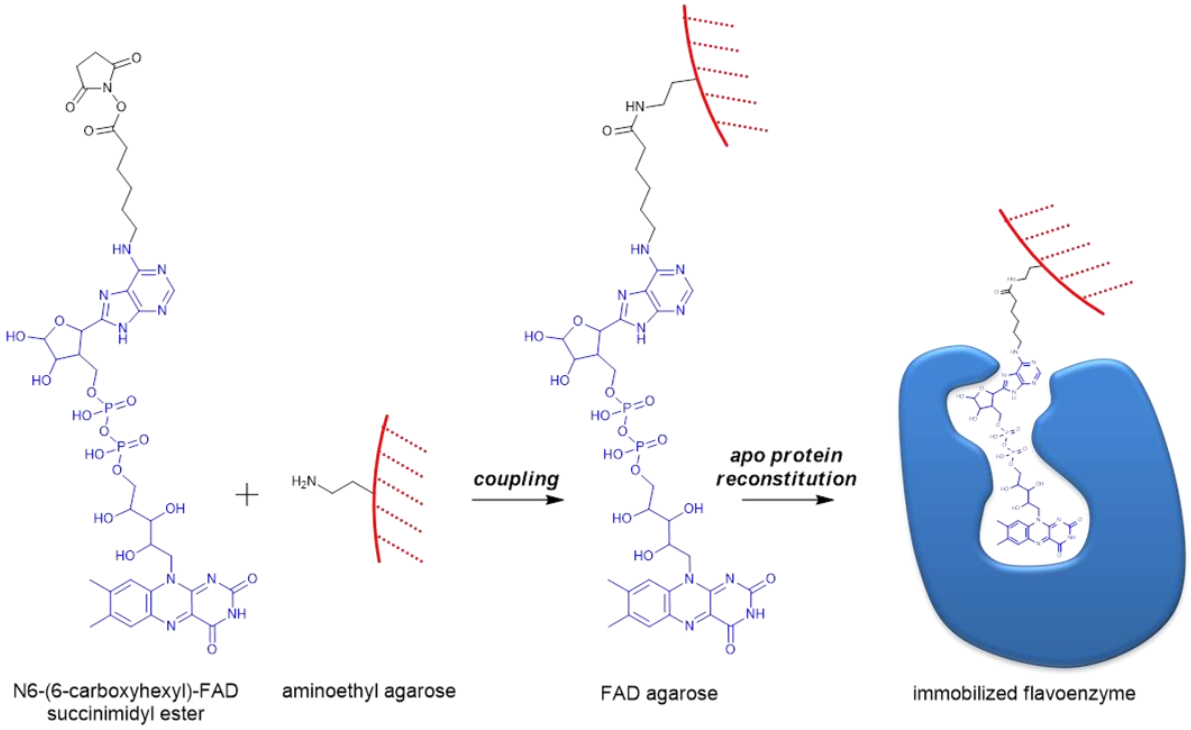

Figure 6. *Example of cofactor-mediated covalent immobilization. The modified FAD cofactor, N^6-(6-carboxyhexyl)-FAD succinimidyl ester, is first covalently immobilized on a carrier and subsequently apo enzyme is reconstituted yielding immobilized holo flavoenzyme.*

3.3 SYNTHESIS OF N^6-SUBSTITUTED FAD ANALOGUES FOR COFACTOR-MEDIATED IMMOBILIZATION

FAD-mediated immobilization requires flavin cofactor derivatives which contain a linker for covalent anchoring to material while it still should bind to the target apo protein. Most commonly it involves incorporation of a primary amine or carboxyl group. Nevertheless, when considering the relatively large and complex FAD molecule (Figure 1), it is clear that a selective derivatization of FAD might be difficult as it contains multiple reactive groups. Besides that, FAD is soluble in aquatic solutions but

poorly soluble in organic solvents and only stable at neutral pH. For these reasons, the scope of chemistry that can be used for modifying FAD is limited and the synthesis of FAD derivatives is very challenging. The most suitable FAD moiety for derivatization is the adenine N^6 as it is usually located close to or at the protein surface (Massey, 2000). This target moiety can be substituted either directly, or first via substitution of the adenine N^1, which subsequently undergoes Dimroth rearrangement into a more stable, N^6-FAD derivative (Bückmann, Wray and Stocker, 1997). FAD analogues synthetized via direct substitution of the adenine N^6 cannot be selectively prepared from the FAD molecule. Therefore, they are synthetized from N^6 functionalized adenosine and FMN, which results in a lengthy multistep and inefficient synthetic pathway (Saleh, Compernolle and Janssen, 1995). Till now, there are only few reports describing methods for chemical FAD derivatization involving a Dimroth rearrangement. In Chapter 3 we describe a new and more efficient pathway for FAD N^6-derivatization.

4. SUMMARY

Flavoenzymes can be used for a wide range of different biocatalytic applications. For their use, efficient immobilization techniques are very valuable. This will allow effective usage of these potent biocatalysts and it may also help in developing new biotechnological tools, such as biosensors in which the flavoenzyme is immobilized on electrodes or efficient flow reactors. By exploiting the tight binding of the flavin cofactor, apo flavoenzymes can be subtly immobilized on any material that has been decorated with FAD. Chapter 2 describes a successful example of such FAD-mediated flavoenzyme immobilization approach. It yielded an immobilized monooxygenase that displayed a higher stability when compared with the enzyme when in solution while it was fully active. As the immobilization is based on binding to the FAD derivatives, the method prevents formation of multilayer of immobilized enzymes. As the newly developed method depends on the availability of reactive and well-designed FAD derivative, effort was put in developing a new synthesis route towards such modified flavin cofactor, derivatized at the adenine N^6. Chapter 3 described such new synthesis protocol which is more efficient when compared with previous reported methods.

A flavoenzyme of significant interest is hFMO3, which catalyzes oxidative metabolism of xenobiotics in human liver. This monooxygenase is of high importance for health-related studies but is very difficult to obtain as isolated enzyme. Chapter 4 describes previous attempts for recombinant expression of this membrane-associated enzyme. Heterologous expression of hFMO3 has been shown to be extremely troublesome. A detailed sequence analysis reveals which parts of the protein may be involved in membrane association and which residues may play a crucial role in catalysis. Furthermore, work is presented on a new attempt of expressing hFMO3: the expression of hFMO3 fused to a coenzyme regenerating enzyme, phosphite dehydrogenase, in *E. coli*. This led to a low level of bacterial expression of active hFMO3. Another and new strategy for generating hFMO-related

metabolites is described in Chapter 5. It is shown that a library of (thermostable) microbial flavoprotein monooxygenases can be used for chemo- and enantioselective oxygenations of known human drugs and metabolites.

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